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# Accepted Manuscript

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# Adrenal Cortical and Chromaffin Stem Cells: Is there a Common Progeny related to Stress Adaptation?

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**Key words:** Adrenal medulla, adrenal cortex, stem cells, progenitors, stress, Nestin

**Abstract**

The adrenal gland is a highly plastic organ with the capacity to adapt the body homeostasis to different physiological needs. The existence of stem-like cells in the adrenal cortex has been revealed in many studies. Recently, we identified and characterized in mice a pool of glia-like multipotent Nestin-expressing progenitor cells, which contributes to the plasticity of the adrenal medulla. In addition, we found that these Nestin progenitors are actively involved in the stress response by giving rise to chromaffin cells. Interestingly, we also observed a Nestin-GFP-positive cell population located under the adrenal capsule and scattered through the cortex. In this article, we discuss the possibility of a common progenitor giving rise to subpopulations of cells both in the adrenal cortex and medulla, the isolation and characterization of this progenitor as well as its clinical potential in transplantation therapies and in pathophysiology.

## 1. Introduction

The adrenal gland is composed of two discrete endocrine organs with grossly distinct embryological origins. The adrenal medulla secreting catecholamines derives from the neural crest, while the adrenal cortex producing corticosteroids develops from the intermediate mesoderm. Both the adrenal cortex and medulla are very dynamic organs involved in the body's adaptation to stress and specific adult progenitor/stem cells are found in both parts, where they contribute to the renewal of organ-specific cells (Ehrhart-Bornstein et al., 2010; Walczak and Hammer, 2015).

We have previously demonstrated the critical role of cellular interactions of chromaffin and cortical cells in adrenal physiology and disease (Bornstein et al., 2008; Ehrhart-Bornstein et al., 1998), and in the rat adrenal medulla we have shown the existence of cortico-chromaffin hybrid cells, i.e. chromaffin cells showing adrenocortical mitochondria and smooth endoplasmatic reticulum typical for steroid hormone producing cells (Bornstein et al., 1991).

Furthermore, we have revealed a direct influence of adrenocortical growth factors and androgens on proliferation and differentiation of both adult adrenomedullary chromaffin cells (Sicard et al., 2007) and chromaffin progenitor cells (Chung et al., 2011). This suggests the involvement of chromaffin progenitors in development and maintenance of the adrenal medulla. However, little is known about interactions between progenitors in the adrenal cortex and medulla, or if to some degree there is a progeny capable of bipotent fates generating both cortical as well as medullary cell types. Alluding to this notion, an extremely rare form of corticomedullary adrenal tumors are characterized by a single mass composed of mixed cell populations with attributes characteristic of both cortical and medullary origin (Michalopoulos et al., 2013). One hypothesis for their origin proposed by Lee et al. suggests a genetic mutation in an adrenal stem cell giving rise to cells with this mixed character (Lee et al., 2008).

## 2. Stem-like cells in the adrenal

### 2.1. Chromaffin progenitor cells

Stem cells are defined as an entity with unlimited self-renewal and the capacity to give rise to multiple cell types (Seaberg and van der Kooy, 2003; Weiner, 2008), while progenitor cells possess limited self-renewal capacity and are often uni- or multipotent. Adrenomedullary chromaffin cells belong to the sympathoadrenal lineage of neural crest derivatives, which also gives rise to sympathetic neurons (Huber et al., 2009). It is still unclear if these two cell types derive from two distinct precursors originating from the neural crest but specified early on in their migration, or from one common bipotential progenitor. Furthermore, it is also unclear, which types of progenitors or stem cells that persist and are maintained in the postnatal adrenal. It has been suggested that a subpopulation of proliferation competent progenitor cells continues to exist in the adult adrenal medulla (Bornstein et al., 2012; Ehrhart-Bornstein et al., 2010). In addition to the adaptation to physiological needs, these progenitor cells may be involved in the development of stress-related hyper activation and hyperplasia of the adrenal and potentially in tumor development (Molatore et al., 2010; Powers et al., 2007).

Within recent years we embarked on isolating chromaffin progenitor cells from the adult adrenal medulla. We have for the first time been able to prove the existence of proliferation and differentiation-competent sympathoadrenal progenitor cells within the adult adrenal medulla and have established methods for their isolation from bovine (Chung et al., 2009; Ehrhart-Bornstein et al., 2010; Masjkur et al., 2014) and human (Santana et al., 2012) adrenals. Under low-attachment cell culture conditions, where adherence to the culture dish is prevented, these cells express progenitor markers, have *in vitro* self-renewing capacity and grow in spheres, which we called chromospheres. Progenitor markers expressed by these cells include Nestin, CD133, Notch 1, Vimentin, Musashi-1, SNAI2, Tumor necrosis factor receptor (NGFR), SOX-9 and SOX-10. *In vitro* these cells are capable of

differentiating into cells of the chromaffin and neural lineages (Chung et al., 2009; Santana et al., 2012; Vukicevic et al., 2012).

## **2.2. Adrenocortical stem cells**

In humans the adrenal cortex is subdivided into three discrete histological and functional layers under the control of distinct endocrine signals: Zona glomerulosa, zona fasciculata and zona reticularis (Xing et al., 2015). Several studies have provided evidence for the existence of adrenocortical cells with stem-like and progenitor-like capacities in various mammalian species like humans, mice, rats or even the common seal (for review see (Walczak and Hammer, 2015)).

Under normal homeostasis adrenocortical stem cells are localized under the organ capsule, where they proliferate and give rise to undifferentiated adrenocortical progenitor cells and differentiated steroidogenic cells. The undifferentiated progenitor cells do not colocalize with steroidogenic markers (Chang et al., 2013). In the zona glomerulosa, the outermost layer, undifferentiated cells are intermingled with the differentiated aldosterone-producing cells in both mice and humans (Heikkila et al., 2002; Nishimoto et al., 2010). The progenitors are displaced centripetally to maintain and adapt adrenocortical function, and finally they undergo apoptosis at the adrenocortical-medullary boundary (Walczak and Hammer, 2015). The regenerative capacity of the adrenal gland has been tested by adrenal enucleation studies, where medulla and most of the cortex was removed leaving behind just the adrenal capsule and the outermost layer of the cortex. Here, a complete restoration of the cortical mass was observed after 6 weeks (Ingle and Higgins, 1938).

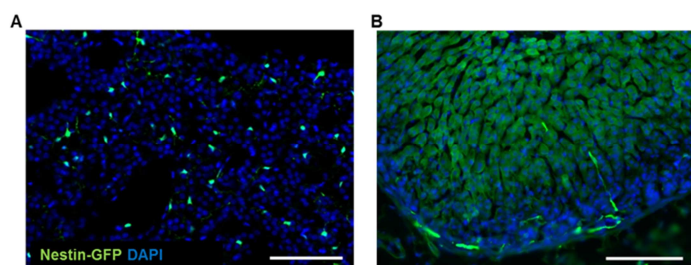
The transcription factor SF1 is an essential regulator of adrenal development and steroidogenic function. The adrenocortical stem cells at the capsule are negative for SF1 and positive for GLI1, a target gene of the Hedgehog signaling pathway. During differentiation into adrenocortical cells these cells become SF1<sup>+</sup>;GLI1<sup>-</sup>, and SF1 then activates the transcription of the steroidogenic enzymes

responsible for catalyzing steroid biosynthesis (Honda et al., 1993; Lala et al., 1992). A negative regulator of SF1-mediated transcription is DAX-1, a factor that has been shown to be essential for maintaining pluripotency (Khalfallah et al., 2009).

### 2.3. Nestin expressing progenitors

The intermediate filament Nestin was initially identified as a marker of neural stem and progenitor cells (Cattaneo and McKay, 1990; Lendahl et al., 1990), but has later been shown also to be expressed in stem/progenitor populations in different adult tissues like for example hair follicles (Li et al., 2003; Sellheyer and Krahle, 2010), skeletal muscle (Birbrair et al., 2011; Day et al., 2007) or pancreatic islets (Zulewski et al., 2001). Therefore, it has been suggested that Nestin expression is associated with a stem/progenitor cell population with multipotent properties and regenerative potential (Wiese et al., 2004).

Since we have previously shown that human and bovine medullary progenitors express Nestin (Chung et al., 2009; Santana et al., 2012; Vukicevic et al., 2012), we were able to take advantage of the Nestin-GFP mouse model, where enhanced green fluorescent protein is expressed under the regulation of the *Nes* gene regulatory element (Mignone et al., 2004). In this mouse we detected Nestin-positive cells through expression of GFP, both in the adrenal medulla and cortex (Fig. 1).

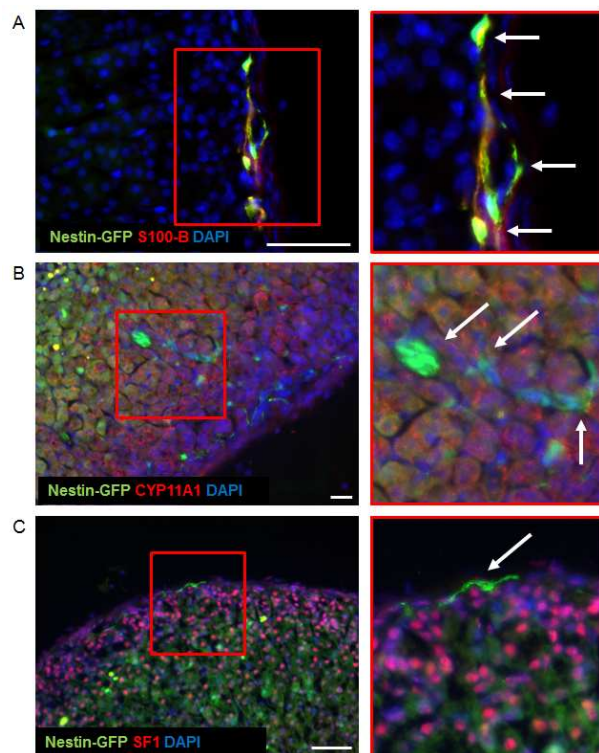


**Figure 1: Nestin-GFP-positive cells in the adrenal medulla and cortex.** (A) Nestin-GFP-positive cells in the medulla were intermingled with clusters of chromaffin cells. (B) Nestin-GFP-positive cells in the cortex were mainly located under the adrenal capsule although some cells appeared to be crossing the cortex with elongated Nestin-GFP-positive processes. Scale bars: 200  $\mu$ m.



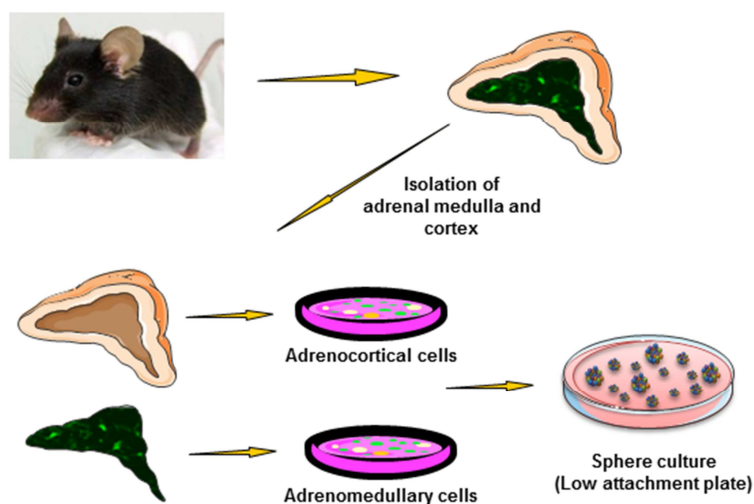
The highest prevalence of Nestin-positive cells was seen in the medulla and in the zona glomerulosa directly underneath the capsule, while a few Nestin-positive cells were scattered in the zona fasciculata and zona glomerulosa. This is opposed to what was previously reported in rats (Bertelli et al., 2002) and humans (Toti et al., 2005), where endogenous Nestin-expression detected by immunocytochemistry was revealed in a subpopulation of adrenocortical cells prevalently located in the zona reticularis, but also some times in the zona fasciculata.

Nestin-GFP cells in both the adrenal medulla and cortex were positive for S100-B (Fig. 2), a calcium binding protein, which is normally expressed in sustentacular glia-like cells of the adrenal medulla suggesting a supporting role for the Nestin-positive cells. However, they were negative for markers of more differentiated cells of the chromaffin lineage like tyrosine hydroxylase or chromogranin A (Rubin de Celis et al., 2015). Furthermore, they were negative for steroidogenic markers like SF1 or CYP11A1 (Fig. 2).



**Figure 2: Nestin-GFP-positive cells in the adrenal cortex.** (A) Nestin-GFP cells located in the cortex were positive for the glia marker S100-B, but negative for the steroidogenic markers (B) CYP11A1 and (C) SF1. Nestin-GFP-positive cells are indicated with white arrows. Scale bars: 50  $\mu$ m.

In order to further characterize the Nestin-positive cells from adrenal cortex and medulla, respectively, we followed the procedure described previously (Rubin de Celis et al., 2015) and outlined in Fig. 3 for the isolation and separation of adrenocortical and adrenomedullary cells.

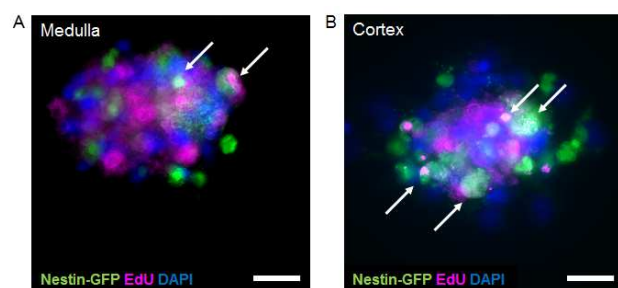


**Figure 3. Isolation of adrenocortical and adrenomedullary cells.** In order to characterize Nestin-GFP cells *in vitro* in culture, the entire adrenal cortex was carefully separated from the adrenal medulla to obtain pure medullary and cortical cultures. Isolated cells from the adrenals of Nestin-GFP mice were cultured under sphere-forming conditions in low-attachment plates, where they generated chromospheres the day after isolation. After 24 h in culture all chromospheres contained Nestin-GFP-positive cells.

All Nestin-GFP cells isolated from either the adrenal medulla or cortex were included in spheres when cultured under low-attachment conditions, which is equivalent to what has been observed with Nestin-GFP cells from this mouse model in other tissues (Birbrair et al., 2011; Day et al., 2007; El-Helou et al., 2013; Li et al., 2003; Mignone et al., 2004; Mignone et al., 2007).

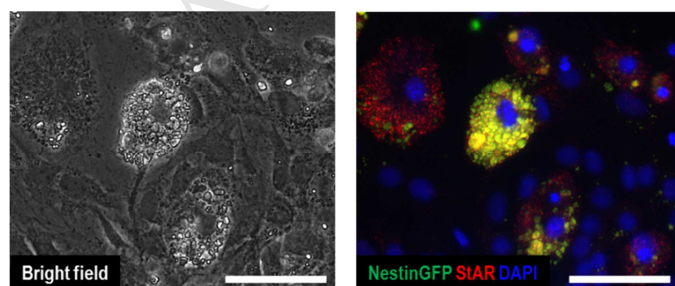
When the spheres were mechanically disrupted and treated with 5-Ethynyl-2'-deoxy-uridine (EdU) for 24 h a part of the cells within the spheres became positive for EdU (Fig. 4) revealing the proliferation and self-renewing capacity of the Nestin-GFP population. The proliferative and sphere-forming capacities of cells from adrenal medulla or cortex are similar, but dependent on the growth media. The highest proliferation was observed with medium optimized for neuronal stem cells (Neurobasal medium with B27 serum-free supplement, Thermo Fisher Scientific) compared to

DMEM/F12 medium containing 10% FBS (Thermo Fisher Scientific) suggesting that Nestin-GFP positive cells from adrenal medulla and cortex preserve their neural stem cell properties.



**Figure 4. Nestin-GFP cells generate spheres and proliferate *in vitro*.** After a few days in culture under low-attachment conditions Nestin-GFP positive cells generated spheres *in vitro*. 24 h treatment with 5-Ethynyl-2'-deoxy-uridine (EdU) revealed incorporation in Nestin-GFP-positive cells from both the (A) adrenal medulla and (B) cortex confirming their proliferative capacity. Nestin-GFP positive cells that have incorporated EdU are indicated with arrows. Scale bars: 20  $\mu$ m.

When free-floating spheres were transferred to plates coated with poly-D-lysine and fibronectin differentiation was promoted for both Nestin-GFP cells isolated from adrenal medulla or cortex, respectively. Progenitor cells from the adrenal medulla were able to differentiate into three different populations *in vitro*; neuron-like cells, glia and chromaffin cells showing the multipotent potential of these progenitors (Rubin de Celis et al., 2015). After differentiation Nestin-GFP expression was reduced. Nestin-GFP positive stem Leydig cells isolated from the testes of postnatal Nestin-GFP mice have been shown capable of differentiating into Leydig cells with the ability to produce testosterone both *in vitro* and *in vivo* (Jiang et al., 2014). Also Nestin-GFP cells isolated from the adrenal cortex seem to be able to differentiate into steroidogenic cells, as Nestin-GFP-positive cells after six days of differentiation contain lipid droplets and become StAR-positive (Fig. 5). Some of these cells still have a high Nestin-GFP expression, whereas other cells with this steroidogenic phenotype show reduced GFP expression.



**Figure 5. *In vitro* differentiation of Nestin-GFP-positive cells into steroidogenic cells.** Nestin-GFP cells isolated from the adrenal cortex plated on poly-D-lysine and fibronectin-coated plates differentiate into StAR-positive cells, which also contain lipid droplets. Scale bars: 50  $\mu$ m

#### 2.4. Nestin expressing stem-like progenitors in tumor development

Cancer stem-like cells belong to a subpopulation of self-renewing cells that are more resistant to chemotherapy and radiation therapy than the other surrounding cancer cells. The cancer stem cell model predicts that only a subset of cancer cells possess the ability to self-renew and produce progenitor cells that can reconstitute and sustain tumor growth under particular conditions.

The gene expression patterns and underlying mechanisms of cells from the sympathoadrenal lineage and neuroblastoma tumor cells show many striking similarities. Many of the molecular and genetic features of cancer stem cells, such as the expression of Sox10 or Snail are similar to those of neural crest progenitors (Hwang et al., 2011; Shakhova et al., 2012) indicating that neuroblastoma tumors are likely to arise from early embryonic neural crest tissues undergoing differentiation and the process of epithelial-to-mesenchymal transition (Louis and Shohet, 2015). Endocrine tumors are relatively uncommon but some studies with cancer-stem-like cells have been performed in endocrine tissues like the thyroid (Nagayama et al., 2016) and the anterior pituitary (Gleiberman et al., 2008; Xu et al., 2009; Yunoue et al., 2011) (reviewed in (Martinez-Barbera and Andoniadou, 2016)).

Recent studies have identified the cell surface marker CD114 (granulocyte colony stimulating factor (GCSF) receptor) as a potential marker for cancer stem cells in neural crest-derived tumors (reviewed in (Zage et al., 2016)). In addition to being present at precursor cells in the bone marrow, CD114 is normally expressed on the surfaces of neuronal and neural crest-derived cell types, where signaling through CD114 promotes neurogenesis and the survival and expansion of neural stem cells (Schneider et al., 2005). However, cells expressing CD114 isolated from neuroblastoma tumors have also been shown to be highly tumorigenic as they demonstrate capability of self-renewal, differentiation and resistance to treatment (Agarwal et al., 2015; Hsu et al., 2013; Zhang et al., 2015). Nestin expression was observed in cortical adenomas and adrenocortical tumors, where Nestin expression correlates with malignancy (Krupkova et al., 2010). Increased Nestin expression has also

been observed in metastatic neuroblastoma when compared to a benign ganglioneuroma and adrenal adenoma (Jensen et al., 2015), and in another study more than 80% of adrenocortical carcinomas stained positive for Nestin, whereas only around 10% of normal adrenal glands and adrenocortical adenomas were Nestin-positive (Lachenmayer et al., 2009).

In pheochromocytomas in the adrenal medulla Nestin immunoreactivity has also been observed but in less than 10% of the tumors (Ehrmann et al., 2005; Oudijk et al., 2015). Since Nestin expression can be related to malignancy and an undifferentiated state of the tumor this suggests that Nestin expression can be the consequence of a failure in certain key regulatory pathways in the stem cells (Krupkova et al., 2010). For example, adrenocortical tumors have been suggested to arise from a Wnt/ $\beta$ -catenin dysregulation (Simon and Hammer, 2012), which is not surprising as the activation of the Wnt/ $\beta$ -catenin pathway is crucial for adrenal development and homeostatic maintenance of the gland (Kim et al., 2008).

### 3. Stress

The adrenal gland has a central role in the body's reaction to stress and the proper adaptation of adrenal function is central in the stress response and regain of homeostasis (Goldstein, 2010).

Response of the endocrine system to stress is characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adrenomedullary system, associated with hypersecretion of adrenal hormones, particularly adrenocortical glucocorticoids and adrenomedullary epinephrine. The reaction of the adrenal gland to different stressors has been extensively studied over the last 20 years in different animal models (for review: (de Celis et al., 2016; Ehrhart-Bornstein and Bornstein, 2008; Kvetnansky et al., 2009)) or in a variety of clinical situations (Goldstein and Kopin, 2008).

Long-term adaptation to chronic stress in addition to hormone release also includes hyperplasia of the gland, involving both the adrenal cortex and the adrenal medulla (Ulrich-Lai et al., 2006). Very

little, however, is known about the adaptation of the adrenal medulla to long-term stress at the cellular level and the involvement of progenitor/stem cells. It is of interest that repeated immobilization stress in rats leads to a dramatic upregulation of transcription factors in the adrenal medulla as shown by gene profiling (Liu et al., 2008). In this study, the most prominently upregulated transcription factor was MASH1, which is transiently expressed in differentiating sympathoadrenal progenitor cells and is crucial for the development of the adrenal medulla (Huber et al., 2002). These data indicate the involvement of sympathoadrenal progenitor cells that persist in the niche of the adult adrenal medulla in the gland's adaptation to chronic stress.

Adaptation under conditions of stress is a priority for all organisms as survival and well-being depends on appropriate physiological responses to environmental and homeostatic challenges. Stress can be broadly defined as an actual (physical) or anticipated (non-physical) disruption of homeostasis or an anticipated threat to well-being. Depending on the type of stressor the body's reaction varies: physical stressors, such as blood loss, respiratory distress, infection or pain require an immediate systemic reaction that is triggered by reflexive mechanisms. Non-physical or "psychogenic" stressors such as immobilization stress are based on prior experiences or innate programs; they require processing in the forebrain (reviewed in (Herman et al., 2016)).

A number of studies addressed the involvement of the adrenal medulla in the body's reaction of non-physical stressors including immobilization stress (Wong et al., 2012) forming the basis for our investigation of the effect of immobilization stress on sympathoadrenal progenitor cells in the medulla. Using the Nestin-GFP mouse model we have shown that immobilization stress promotes the differentiation of Nestin-positive cells in the adrenal medulla (Rubin de Celis et al., 2015). In stressed mice the adrenal glands were enlarged, and the proliferation rate of Nestin-GFP positive cells was increased. At the same time the number of Nestin-GFP positive cells decreased, which suggests an involvement of the Nestin-positive cells in the adaptation of the adrenal gland to stress. Furthermore, lineage tracing using tamoxifen-induced Nes-CreERT/Rosa26-YFP mice (Burns et al.,

2007; Srinivas et al., 2001) under immobilization stress showed that the Nestin positive cells under stress differentiate into chromaffin cells. This further demonstrates that Nestin expressing progenitors in the adrenal medulla differentiate to adapt the gland to physiological needs (Rubin de Celis et al., 2015).

#### **4. Chromospheres as a cell source in transplantation therapies of neurodegenerative diseases**

Parkinson's disease is a neurodegenerative disorder characterized by non-motor and motor symptoms. The physiopathology of Parkinson's disease motor symptoms is associated with the loss of dopaminergic neurons (Braak et al., 2004). As an alternative to medication, cell replacement therapy by grafting dopamine-releasing cells has been proposed (Lindvall and Bjorklund, 2004). Due to the close relation of chromaffin cells to catecholaminergic neurons, a substantial number of studies have promoted the use of chromaffin cells for this purpose (reviewed in (Ambriz-Tututi et al., 2012)).

In animal models of Parkinson's disease, chromaffin cell grafting experiments performed in the 1980s showed moderate but highly variable motor improvements (Fiandaca et al., 1988; Freed et al., 1981; Morihisa et al., 1984), even though only 1% of the chromaffin cells were able to release dopamine (Fernandez-Espejo et al., 2005). However, in humans these cells did not show satisfactory results, as their grafting in patients produced only partial motor improvements (Madrado et al., 1987). Furthermore, these improvements were only transient and highly variable among subjects (Barker et al., 2015). In addition, high morbidity and mortality were associated with this grafting procedure (Barker et al., 2015).

Due to their favorable properties, chromospheres could represent a new and promising source for cell replacement therapy in Parkinson's disease as a significantly higher population of chromosphere-derived cells has been shown to acquire a dopaminergic phenotype compared to chromaffin cells (Boronat-Garcia et al., 2016; Vukicevic et al., 2012).



## 5. Conclusion

The adrenal gland unites two endocrine tissues under one organ capsule. A close morphological and functional interdependence of cortical and chromaffin cells has already been demonstrated (reviewed in (Ehrhart-Bornstein and Bornstein, 2008)). In fact, it becomes more and more clear that the functions of the adrenal gland should be viewed more as an integrated whole greater than the sum of its parts (Haase et al., 2011; Vinson, 2016).

Despite the different developmental pathways of the two adrenal areas, their progenitor populations express some common genes; a Nestin-positive progenitor population exists in both the adrenal medulla and cortex, and the proliferation capacities and the expression patterns of these cells under basal conditions are very similar. Therefore, the level of plasticity in adrenal development and function is more complex than hitherto assumed, suggesting to some degree an overlapping progeny of subpopulations of cortex and medulla.

Supported by the existence of cortico-chromaffin hybrid cells (Bornstein et al., 1991) and mixed corticomedullary tumors (Michalopoulos et al., 2013), we here challenge the dogma of a strictly separated progeny of the two systems. Because of the rarity of mixed adrenal tumors and thereby limited amount of literature describing this, it is still not possible to say if they derive from a common precursor and further investigation will be needed in order to solve this question. However, elsewhere in the endocrine glands of the HPA axis there is increasing evidence for a common precursor giving rise to mixed tumors. In mixed pituitary adenomas containing gangliocytoma, which are composed of adenomatous and neuronal components, the presence of neurofilament (NFP) in pituitary adenomas indicates neuronal differentiation in adenoma cells (Kontogeorgos et al., 2006). This suggests a common stem/progenitor cell origin of both the adenomatous and neuronal component of these lesions, which also might be the case in mixed adrenal tumors.



In addition to or instead of its potential role in generation of steroidogenic cortical tissue the Nestin-positive cell population in the adrenal cortex might also play a role in maintenance of cortical zones by secretion of adrenocortical growth factors. Another possible role could be the secretion of androgens known to influence the proliferation and differentiation of progenitors and chromaffin cells in the adrenal medulla. For example, different androgens produced in the adrenal cortex can induce enzymes necessary for catecholamine biosynthesis, like glucocorticoids that induce the phenylethanolamine N-methyltransferase (PNMT) converting norepinephrine to epinephrine (Wurtman and Axelrod, 1965), or dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S) that induce the tyrosine hydroxylase (TH) metabolizing tyrosine to dihydroxy-phenylalanine (L-DOPA) (Charalampopoulos et al., 2005).

Although they show a very low proliferative rate under basal conditions, in stress situations the sympathoadrenal progenitors seem to be able to differentiate towards the chromaffin cell lineage, also indicating a supportive role for these cells under stress.

However, to decide if the Nestin-positive adrenocortical cells belong to the same cell lineage as the Nestin-positive sympathoadrenal progenitors, their origins, differentiation potential and the role of adrenocortical progenitors under stress still have to be further elucidated. These findings can introduce new concepts and therapeutic potential in the field of adrenal research.

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**Highlights**

- Nestin-positive progenitors persist in both the adrenal medulla and cortex.
- Sympathoadrenal progenitors differentiate to chromaffin cells under stress
- Nestin is expressed in adrenal tumors and associated with tumor malignancy
- Chromospheres might be used for transplantation therapies of neurodegenerative diseases